https://dx.doi.org/10.21577/0103-5053.20220095

J. Braz. Chem. Soc., Vol. 34, No. 1, 135-145, 2023

Article

Honey of *Tetragonisca angustula* from Southwestern Bahia: Influence of Seasonality on the Physicochemical Profile and Glioma Cell Inhibitory Effect

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This study aims to analyze *Tetragonisca angustula* honey from southwestern Bahia for its physicochemical characteristics, phenolic compounds and glioma cell inhibitory effect, in samples from different periods of the year, dry (DP) and rainy (RP) period. The analyses showed that samples obtained in the DP and RP are statistically different for the physicochemical profile ($p < 0.05$). DP honey has a darker color, greater acidity, content of reducing sugars and ash and lower moisture, when compared to RP. The content of total phenolics and flavonoids was determined spectrometrically, using the Folin-Ciocalteu method and reaction with aluminum chloride, respectively, and there was no statistical difference between the samples ($p > 0.05$). For the extraction of phenolic compounds, dispersive liquid-liquid microextraction was used, besides analysis by high-performance liquid chromatography with ultraviolet detection (HPLC-UV), which identified chlorogenic acid and quantified caffeic acid and rutin (1.7723 and 0.9384 μg 100 mg^{-1} honey, respectively). Glioma cell inhibition effect lines were observed for DP at concentrations 1000 ng mL^{-1} of honey. Principal component analysis (PCA) proposes the physicochemical parameters as the main distinguishing factors between the DP and RP samples. DP samples showed similarity for the parameters ash content, acidity and reducing sugars and the samples of the RP had consonance between pH and moisture.

Keywords: *Tetragonisca angustula*, honey properties, phenolic compounds, glioma cells inhibition, DLLME, PCA

Introduction

Tetragonisca angustula, known as Jataí, "*maria-seca*", indigenous bee and golden bee, is a species of small stingless bee (4 to 5 mm) that has social characteristics and ease of adaptation in different environments. Bees are recognized as one of the most important insects for environmental balance, for acting as a natural pollinator of various plants and collaborating with the maintenance of the biodiversity of different terrestrial ecosystems.[1-3] In addition to pollinating, the *Tetragonisca angustula* bee contributes to meliponiculture by providing honey, with high nutritional value, pollen, propolis and geopropolis.[4]

Honey is considered a viscous fluid, consisting of a complex mixture of nutrients, mainly sugars and, at lower concentrations, organic acids, minerals, vitamins, pollen grains, waxes, phenolic compounds, among other phytocompounds.[5-7] Since the most remote times, this product has been used for food and therapeutic purposes. The chemical composition, aroma and flavor of honey are associated with the environment, pollination process, climatic and geographical variability, plant species available for propolis collection and honey bee species.[8,9] Its chemical composition and concentrations of the constituents that comprise it are responsible for the therapeutic potential of

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Editor handled this article: Eduardo Carasek

this product.[10] Studies[11,12] indicate phenolic compounds of different classes (phenolic acids, flavonoids and organic acids) and protein as responsible for the antioxidant, anti-inflammatory, cardioprotective and antitumor action.

The Brazilian semiarid region is characterized by a dry climate with high temperatures and soils with low moisture retention capacity. In general, half of the rainfall percentage is concentrated in the wettest quarter (wet period), which is characteristic of summer months. On the other hand, the dry period extends, on average, for 6-8 months.[13] The characteristic biome of the semi-arid region is the Caatinga, and all biological heritage in this region is exclusively Brazilian.[14] Adverse climatic and geographical conditions result in the resistance of honey plants and their admirable recovery power.[15]

The multivariate method of principal component analysis (PCA) is the technique commonly used in analysis to classify foods. The aim of PCA is to determine the lower dimensional space ensuring the greatest level of variability. Therefore, new linear functions of independent variables are generated in the original dataset, known as principal components (PCs). The most significant percentages expressed by the PCs indicate the variables with the greatest contribution to the variation of the analyses. Thus, it is possible to visualize the structure of the data and identify probable groupings.[16-18]

The Brazilian Northeast has great potential for meliponiculture, due to the biodiversity, presence of native plant species and climatic variability. Considering that such factors influence the physicochemical characteristics and bioactive composition of honey, this study aimed to analyze *T. angustula* honey in terms of its physicochemical characteristics and phenolic compounds, correlating them through PCA, in samples from different periods of the year, dry (DP) and rainy (RP) periods, in the Southwestern region of Bahia, in addition to the quantification of phenolic compounds and analysis of the inhibitory effect of murine glioma cells in a DP sample.

Experimental

Materials

All chemicals and reagents used were of analytical grade. Deionized water, obtained from a deionizing system (Milli-Q®, Millipore, USA) with a resistivity of 18.2 MΩ cm, was used throughout all experiments. The reference standards were from Sigma Aldrich: quercetin (Ref. No. Q4951, CAS No. 117-39-5, St. Louis, MO, USA with purity of ≥ 95%), rutin (Ref. No. PHL89270, CAS No. 153-18-4, São Paulo, Brazil), caffeic acid (Ref. No. C0625, CAS No. 331-39-5, St. Louis, MO, USA), chlorogenic acid (Ref. No. C3878, CAS No. 327-97-9, São Paulo, Brazil), *trans*-ferulic acid (Ref. No. 128708, CAS No. 537-98-4, São Paulo, Brazil), *p*-coumaric acid (Ref. No. C9008, CAS No. 501-98-4, São Paulo, Brazil) and gallic acid (Ref. No. G7384, CAS No. 149-91-7, São Paulo, Brazil). The ultrapure solvents used in the high-performance liquid chromatography with ultraviolet detection (HPLC-UV, CBM-20A, Shimadzu Co., Kyoto, Japan) were methanol (Ref. No. 34860, CAS No. 67-56-1, São Paulo, Brazil, from Sigma-Aldrich) and acetic acid (Supelco, Bellefonte, USA). The Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum and penicillin 10,000 IU L^{-1}, streptomycin 10,000 μg mL^{-1} (Gibco, Grand Island, NY, USA) were also used.

Sample collection

T. angustula honey samples were collected directly from stingless bees, on the Faustino Farm, a rural area in the municipality of Rio do Antônio, Bahia, Brazil (14º21'28.36" S 42º00'01.48" W) (Figure 1), registered in SisGen with code AE21C0B. The samples were packed in sterile bottles and refrigerated. The honey samples were obtained in two distinct periods: dry period (DP)-October 2018; and rainy period (RP)-February 2019. The climate recorded in Rio do Antonio in the months of collection were: relative humidity average-59.9% (October) and 65.0% (February); average rainfall-23.78 mm (October) and 91.10 mm (February).[19]

Physicochemical characteristics

The physicochemical characteristics of the honey samples collected in DP and RP were as follows: pH, free acidity, moisture, reducing sugars, ash content and color. Analyses were carried out following the protocols suggested by Institute Adolfo Lutz,[20] Laboratório Nacional de Referência Animal (LANARA-MAPA)[21] and the Association of Official Analytical Chemists,[22,23] in triplicate and with modifications.

Free acidity and pH

pH measurements were determined based on the Association of Official Analytical Chemists,[22] using a digital potentiometer (DM-22, Digimed, São Paulo, Brazil). The solution was prepared with 2 g of honey samples diluted in 15 mL of distilled water.

Free acids were quantified by titration with standardized sodium hydroxide (NaOH) solution at 0.1003 mol L^{-1}, until it reached pH 8.3; the volume of NaOH spent was recorded

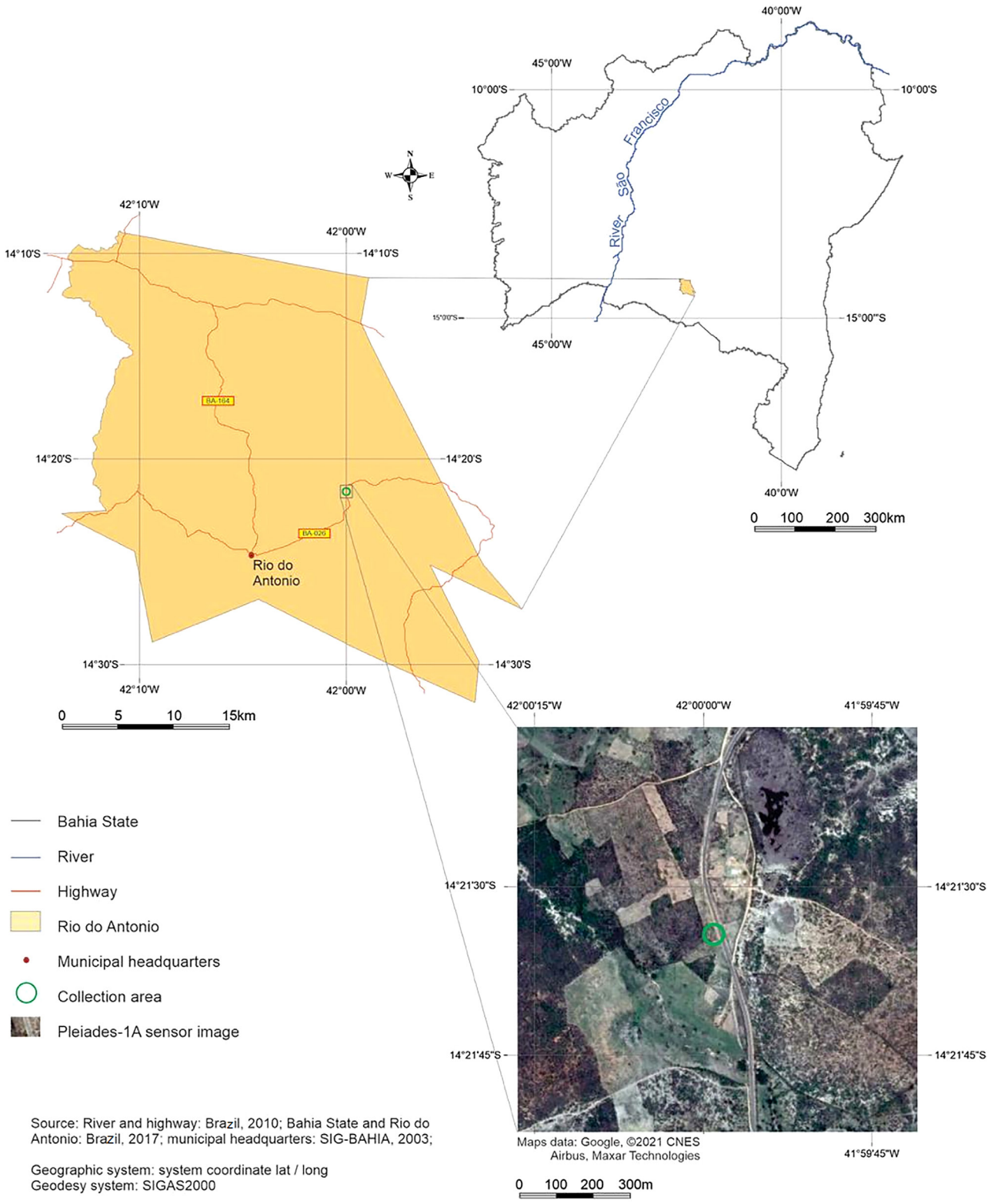

Figure 1. Map of Bahia region with emphasis on the honey sample collection area.

in the titration. Honey acidity, in meq kg^{-1}, was calculated using the equation 1:

$$\text{Acidity} = V_b \times M \times 1000/m \quad (1)$$

where, V_b: volume of sodium hydroxide spent at the end of titration; M: molar concentration of base and m: mass of honey used in the solution.

Moisture

Moisture was recorded directly from the honey samples, without dilution, and read in a portable Abbe digital refractometer (Germany) (natural light and room temperature). Moisture was calculated by converting the corrected refractive index value using the Chataway table, based on the AOAC method.[22]

Reducing sugars

Reducing sugars were determined using the method described by the LANARA.[21] The determination of reducing sugars consists in the reduction of Fehling solutions A (copper sulfate pentahydrate at a concentration of 0.070 g mL^{-1}) and B (sodium and potassium tartrate, at 0.35 g mL^{-1}) with a solution of reducing sugars from honey.[21] The Fehling solutions were previously standardized from the titration of the aqueous glucose solution (0.5 g of glucose *per* 100 mL of water) with methylene blue as an indicator (1%). The solution title was calculated using the equation 2:

$$\text{Title} = V_{glucose} \times m_{glucose}/100 \quad (2)$$

where $V_{glucose}$: volume of the glucose solution (mL) used in the titration and $m_{glucose}$: mass (g) used in the preparation of the solution.

For the analysis, approximately 2.5 g honey were weighed and transferred to a 500-mL volumetric flask. The standardized Fehling solutions A and B were transferred to a 250-mL flask containing 7.0 mL of water and 15 mL of honey solution, with methylene blue as an indicator (1%). The percentage of reducing sugars was expressed using the equation 3:

$$\text{Reducing sugars (\%)} = 100 \times A \times T/m \times V \quad (3)$$

where A: burette volume; T: solution title; m: honey mass; and V: volume of titrant used.

Ash

The principle involved is that, when a known weight of feed is ignited to ash, the weight of ash obtained is expressed in terms of percentage. To obtain the percentage of ash, 1 g of honey samples was dried and calcined at 550 ºC for 4 h. Finally, the remaining ashes were weighed and the content was expressed in g *per* 100 g of honey using the equation 4:

$$\text{Ash (\%)} = 100 \times N/m \quad (4)$$

where N: ash mass in grams and m: mass of the sample.[20,21]

Color

The method to determine the color of honey was based on the different degrees of light absorption, through a UV-Vis spectrophotometer (Shimadzu UV-1601 UV/VIS, Japan) at 635 nm, using glycerin as a blank. Absorbance (Abs) was determined and the value of mmPFund was obtained using the following equation 5. The classification of color intensity followed the Pfund scale.[24-26]

$$\text{mmPFund} = -38.70 + 371.39 \times \text{Abs} \quad (5)$$

Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method.[27] In test tubes, an aliquot of 0.5 mL of the honey solution (0.1 g mL^{-1} in distilled water), 0.3 mL of the Folin-Ciocalteu reagent and 2 mL of the sodium carbonate solution were mixed (15%) and 2.2 mL of distilled water were added. The system was then incubated for 2 h at 40 °C and absorbance was read in a spectrophotometer at 798 nm. To calculate the content of phenolic compounds, a calibration curve was plotted with the gallic acid standard, at concentrations of 0.02 to 0.2 mg L^{-1}. The results were expressed as milligram of gallic acid equivalent *per* kilogram of honey (mg GAE kg^{-1}).

Total flavonoids content

The concentration of flavonoids present in the honey sample was determined by a spectrophotometric method, using aluminum chloride (5%), and the results were expressed in milligrams of quercetin equivalent *per* kilogram of honey (mg QE kg^{-1}). In test tubes, 2-mL aliquots of the honey solution (0.1 g mL^{-1} in distilled water) were placed with 3 mL of aluminum chloride (5%), followed by incubation for 30 min and reading at 437 nm, with methanol as a blank. To calculate the content of flavonoids, a calibration curve was plotted with the quercetin standard, at concentrations of 8.0 to 50 mg L^{-1}.[27]

Extraction and chromatographic analysis

Phenolic compounds for HPLC analysis were extracted from honey samples for the dry period through dispersive liquid-liquid microextraction (DLLME), based on the method adapted by Campone *et al.*[28] This technique allows the preconcentration and optimization in the extraction of phenolic compounds. The fractions obtained were used in HPLC-UV analysis and for *in vitro* evaluation of glioma cells growth inhibition.

Phenolic compounds were identified by reverse phase HPLC using a C18 analytical column (Supelco analítica, 25 cm × 4.6 mm × 5 μm) at a constant temperature of 25 ºC. The mobile phase of ultrapure water acidified with

0.1% acetic acid (A) and also with acidified methanol (B), using a flow of 1.0 mL min^{1}. The elution gradient was as follows: 25 to 100% B from 0-40 min and 100% B from 40 to 50 min. The solutions were filtered using a 0.45-µm membrane filter (polytetrafluoroethylene (PTFE)) and injected manually (30 µL) with a microsyringe. The analysis of phenolic compounds was performed using a UV detector, with the wavelength defined at 254 nm. Caffeic, *p*-coumaric, chlorogenic, gallic and *trans*-ferulic acids were identified, in addition to the flavonoids rutin and quercetin; quantification was performed for the substances caffeic acid and rutin. The identification was determined by comparison of the retention time of the sample components with the spectra of the standards and co-injection of standard and sample. For quantification, an analytical curve was plotted by averaging the absolute peak area *versus* concentration, using the standards separately, in triplicate, with six points ranging from 1.0 to 10.0 µg mL^{-1}.

Glioma cell growth inhibition assay

The inhibitory effect was analyzed with the DP sample on murine glioma cell line (C6), from the American Type Culture Collection, cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 100 µg mL^{-1} streptomycin and 100 µI mL^{-1} penicillin in 10-cm culture plates (TTP, Switzerland). The cells were seeded in 96-well plates at a density of 3.1×10^4 cells cm^{-2} and treated with DP samples at doses of 100-15000 ng mL^{-1}. The negative control was evaluated in the absence of the honey sample. The plates were treated under ambient conditions with 5% CO_2, at 37 °C, for 72 h. After this time, the culture medium was replaced by one containing 1 mg mL^{-1} of MTT (3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide) and lysed with lysis buffer containing 20% (m/v) sodium dodecyl sulfate (SDS) in 50% (v/v) *N*,*N*-dimethylformamide, at pH 4.7. The analyses were performed after 24 h, by spectrophotometry at 595 nm (Varioskan Flash™, Waltham, USA).

Statistical analysis

The results obtained were evaluated by analysis of variance (ANOVA) to compare the means, followed by the Tukey's test. The correlation coefficients (r) were obtained at a significance level of 0.05. Principal component analysis (PCA) was used to investigate the correlations between physicochemical characteristics (pH, acidity, moisture, reducing sugars and ash) and phenolic compounds in the two collection periods. The software adopted was the Statistica 12.0 (Stat Soft Company).[29]

Results and Discussion

Physicochemical analysis

The analysis of the physicochemical properties of honey are important, since these properties are influenced by factors such as soil typology, type of flowering, climatic conditions and bee species. The results in relation to the physicochemical parameters evaluated in *T. angustula* honey for DP and RP samples are shown in Figure 2. There was a statistically significant difference ($p < 0.05$) between honey samples for the parameters: pH, free acidity, moisture, reducing sugars, ash content and color. Only the content of phenolic compounds and flavonoids did not differ significantly ($p > 0.05$); for this reason, the HPLC-UV analysis followed with the dry period samples.

The DP sample showed greater acidity (97.13 ± 5.77 meq kg^{-1} of honey) when compared to RP (69.79 ± 5.77 meq kg^{-1} of honey), a statistically significant difference ($p < 0.05$) of 27.34 meq kg^{-1} honey, (Figure 2b). Acidity and pH are parameters conditioned to the floristic composition of the region, flower pollen and soil conditions. Previous studies showed a wide acidity range in stingless bee honeys, ranging from 25,0-592,0 meq kg^{-1} of honey,[1] while the species *T. angustula* presented acidity of 9.6-66.7 meq kg^{-1} of honey.[30] The results indicate that stingless bee honey seems to exhibit a higher acidity profile than *Apis mellifera*.[30]

Considering these factors, the pH also varied significantly between DP (3.62 ± 0.01) and RP (4.14 ± 0.03) honey samples. de Sousa *et al*.[31] analyzed honeys of Meliponinae in northeastern Brazil in dry and humid periods and found pH 3.60-5.30 which also showed statistical differences between samples.[31] We can suggest that the Bahia semiarid consists of plants that are highly resistant to water stress due to the long drought period, which enables the increase in the concentration of organic acids in plants and reduction in the chemical diversity of their composition, thus influencing the more acidic composition of the collected material during this period. In the humid period, it is possible to observe the diversity of chemical composition and flower supply, superior to that of the dry period. Thus, more classes of compounds enrich the chemical composition of honey, consequently favoring an increase in pH and a reduction in acidity.[31-33]

The RP sample had a higher moisture content. Although short, the humid period with high rainfall in the region provided an accentuated presence of water in the honey samples of that period. According to climatological data from the Meteorological Database for Teaching and Research of the Instituto Nacional de Meteorologia (INMET),[19] the

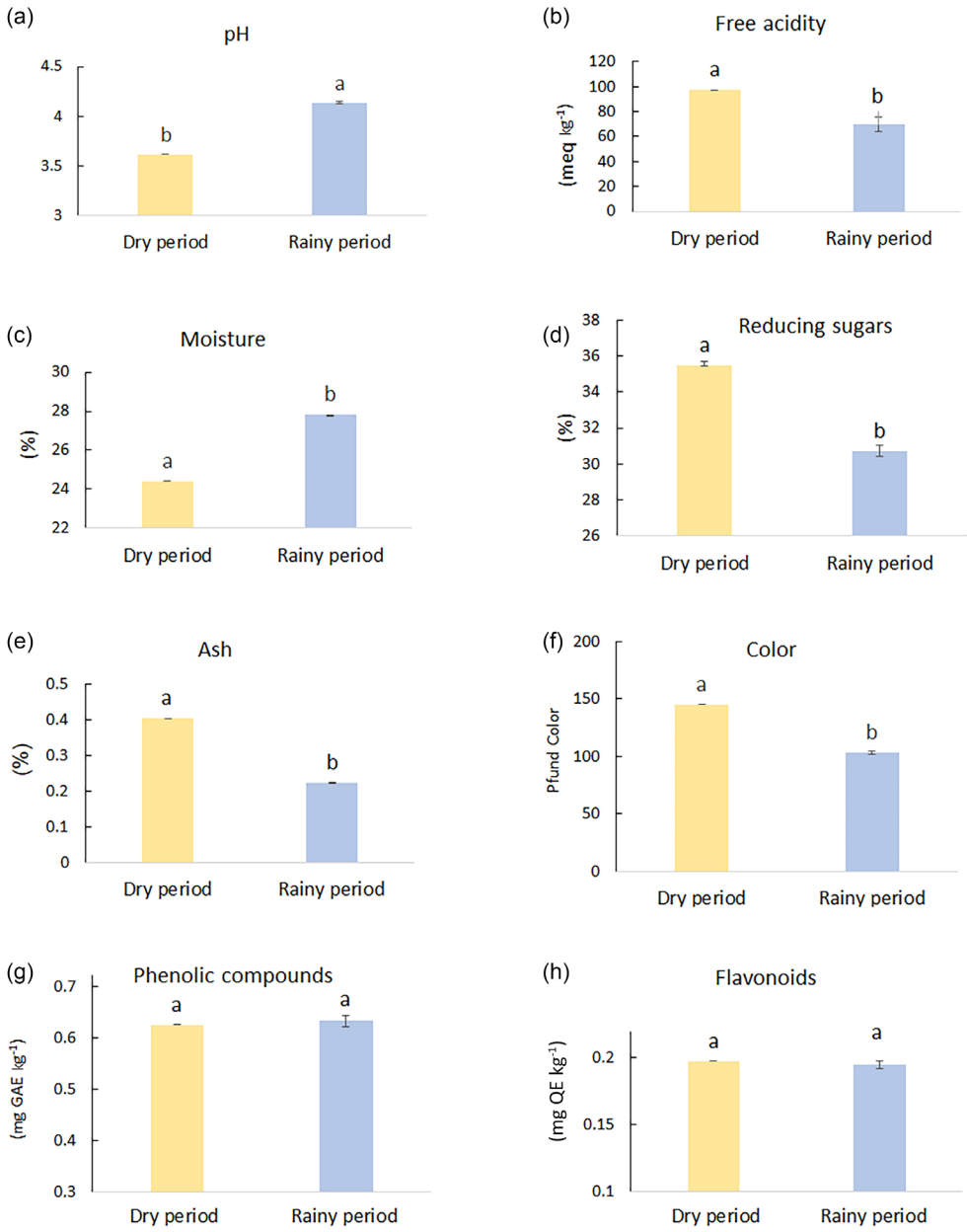

Figure 2. Analytical parameters obtained for honey during the dry period (DP) and rainy period (RP). (a) pH measured in digital pHmeter; (b) free acidity expressed as meq kg^{-1} of honey; (c) moisture percentage; (d) reducing sugar percentage; (e) ash percentage; (f) color expressed as Pfund scale; (g) total phenolic compounds expressed as mg gallic acid equivalents *per* kg of honey (mg GAE kg^{-1}); (h) flavonoids expressed as mg quercetin equivalents *per* kg of honey (mg QE kg^{-1}). All values are presented as a mean ± standard deviation and different letters symbolize significant differences. Student's *t*-test. $p \leq 0.05$.

average value of total rainfall for the dry period (March to October 2018) was 23.78 mm and the wet period (November to February 2019), 91.10 mm, showing a wide variation in the water volume, which caused a difference of 3.41% in moisture analysis between the two periods in the region, as evidenced in Figure 2c (DP 24.40 and RP 27.81%). A similar range of moisture content was observed by Biluca *et al.*[34] 23.1-43.5% for stingless bee species in Brazil and Mokaya *et al.*[35] 26.1-35.9% in the African continent.[34,35]

Moisture content is an extremely important characteristic in honey and food products in general, as it influences product preservation process. For example, the fermentation process and the growth of microorganisms are favored in humid environments with a tendency to spread and survive for long periods. In addition, flavor and crystallization can also be affected by the amount of water present in honey.[4,35] Tropical environments, ripe fruits and bee species are factors that can influence the moisture content of the product.[36]

Despite the variation of 4.76% in the percentage of reducing sugars, the results differed significantly between DP and RP samples, 35.45 and 30.69%, respectively.

The dark color of DP is associated to its higher content of reducing sugars. Previous studies[37,38] have shown a directly proportional relationship between sugar content, ash content and honey color intensity. Stingless bee honey generally has a lower content of reducing sugars than stinging honey, although its flavor is sweeter.[8] This is linked to fructose content, which is mainly responsible for this characteristic. Factors such as botanical origin, climatic conditions and geographical origin contribute to the variation in sugar content in honey.[18] Its predominance in honey composition provides the sweetest flavour.[39]

The DP sample showed a higher percentage of ash (0.4042 ± 0.0013) with a significant difference in relation to the RP sample (0.2247 ± 0.0030), (Figure 2e). Factors such as high reducing sugar content, mineral content and dark coloring can influence ash concentration in the honey sample,[37] as well as collection practices of honey producers, separation processes (decantation and/or filtration) in the final obtention stages of the product, botanical origin and geographical origin of honey.[40,41]

DP honey samples (0.4946 ± 0.0036 nm ABS) was classified as dark color (referring value 145 of the Pfund scale) and RP (0.3823 ± 0.0028 nm ABS) received an amber color classification (exhibiting value 103 according to the Pfund scale). The evident and statistically different color profile between honey samples produced and collected in the dry and wet periods endorses the distinction between the samples (Figure 2f). Flower species, region where they are located, pollen grain type, mineral content, type of phenolic and flavonoid, and other factors influence the final color of the honey.[42] The sharp difference between climatic conditions in the semiarid region makes it possible to change honey color.

Total phenolic content

The phenolic content in the honey samples did not differ statistically ($p > 0.05$) between DP and RP, presenting a concentration value of 0.6248 (± 0.0112) and 0.6321 (± 0.0308) mg GAE kg^{-1}, respectively, as shown in Figure 2g. As well as phenolic constituents, flavonoids also showed no significant differences between samples DP (0.1978 ± 0.0027 mg QE kg^{-1}) and RP (0.1950 ± 0.0012 mg QE kg^{-1}) samples.

Climatic factors and botanical origins are the main factors that justify the low content of phenolics and flavonoids in the analyzed honey samples.[43,44] Despite being incipient, pollen studies using stingless bee honey, conducted in the semi-arid region of Bahia, indicated mostly plants of the families Mimosaceae, Myrtaceae, Poaceae, Euphorbiaceae and Sapindaceae.[45-47]

Using multivariate analyses, Avila,[48] evaluated the influence of the botanical origin of pollen on the properties of stingless bee honey. According to the parameters evaluated, the predominant pollens Sapindaceae and Lamiaceae, characterized the lowest values for total phenolic content (3.00 mg GAE kg^{-1}).[48] The correlation between pollen types and the low phenolic content of stingless bee honey suggests that this aspect is inherent to the floristic composition available in the region. Therefore, our article stands out for developing a pioneering study of preliminary chemical characterization of the *T. angustula* honey from the southwest region of Bahia and collaborates with additional and species-specific information.

The analysis of phenolic compounds by HPLC-UV occurred by the joint analysis of factors: comparison of the retention time of the reference standards analyzed separately, chromatographic profile of the mix solution containing all standards and co-injection of the internal standard. Chromatographic analyses showed the presence of caffeic acid, chlorogenic acid and rutin. There was an increase in peak height at a retention time of 22.970 min and absorbance compatible with that observed for the compound caffeic acid. Similarly, an increase in peak height at a retention time of 18.638 and 20.735 min allows the confirmation of the presence of chlorogenic acid and flavonoid glycoside rutin, respectively.

According to chromatographic analysis, it was not possible to identify gallic acid or the flavonoid quercetin, allowing to infer the absence of these phenolic compounds in the studied *T. angustula* honey sample or the techniques applied in this study did not allow their detection. For *p*-coumaric and *trans*-ferulic acids, the same retention time was observed, at 29.472 min, for the analysis of standards, in the sample and in the co-injection. It is not conclusive as to the presence of just one or a mixture of them.

Caffeic acid and rutin were quantified in the DP sample of *T. angustula*. The calibration curve was plotted with the solutions of the reference standards, separately, at 1, 3, 5, 6, 8 and 10 µg mL^{-1}, in triplicate. From the calibration curves, the respective determination coefficients (R^2) obtained were above 0.992 and the concentrations for caffeic acid were 1.7723 µg of caffeic acid 100 mg^{-1} honey and, for rutin, 0.9384 µg rutin 100 mg^{-1} honey. The limits of detection (LOD) and quantification (LOQ) demonstrated that the methodology is capable of quantifying these substances at low concentrations. For caffeic acid, a LOD of 0.0055 and LOQ 0.017 µg 100 mg^{-1} were observed and, for rutin, it was 0.011 and 0.034 µg 100 mg^{-1}, respectively.

Comparing the phenolic compounds analyzed in this study in *T. angustula* honeys from the semi-arid region of northeastern Brazil with samples from the southern

region of Brazil, it is possible to verify how the geographic conditions and the floristic arrangement influence the chemical composition of honey and not only bee species. Table 1 presents the results obtained in this study (sample D), for analysis of the quantification of phenolic compounds: caffeic, chlorogenic, *p*-coumaric, *trans*-ferulic acids and flavonoids rutin and quercetin, correlated with previous studies conducted in three samples from Florianópolis-Brazil (sample A, Biluca *et al.*[43]); (samples B and C, Biluca *et al.*[49]).

T. angustula bees exhibit behavior generally adopted to obtain their pollen resources and adapt to temporal dynamics of flowering and floral availability at different times of the year.[3] Although the honey samples are from *T. angustula*, it is not possible to observe consistency in the chemical composition of the samples, even those from the same region (samples A, B and C) (Table 1). For this factor, other variables must be considered, including the plant species visited by bees and those that produce pollen and nectar (products from plants and flowers that synthesize bioactive metabolites and are possibly transported to honey in its production process); climatic factors (rainfall level, insolation and temperature); the foraging behavior of bees and the specific needs of each colony.[46-49]

In pollen studies with *T. angustula* conducted in the semiarid region of northeastern Brazil, de Novais *et al*.[46] considered the species *Anadenanthera colubrina*, *Poincianella pyramidalis* and *Ziziphus joazeiro* as flora markers of the region; and the most frequent pollen types in the region species such as *Heteropterys*, *Milania*, *Myrcia*, *Mimosa tenuiflora* and *Solanum*, *Schinus*, *Senna macranthera*, *Chamaecrista*, *Syagrus coronataos* and *Prosopis juliflora* (which is not considered endemic to the Caatinga, but exhibits forage characteristics and resistance to long drought periods).[46,47,50,51]

The region of Rio Antônio-Brazil has native species of the Caatinga (dry vegetation) such as umbuzeiro (*Spondias tuberosa*, Sapindaceae), black jurema (*Mimosa tenuiflora*), juazeiro (*Ziziphus joazeiro*) arueira (*Schinus terebinthifolius*) and liana flower, species visited by stingless bees. Pollen studies recorded in the literature for semi-arid regions of northeastern Brazil indicate the predominance of native plants of the families Amaranthaceae, Anacardiaceae, Fabaceae, Malvaceae (Herissantia, Waltheria), Moraceae, Rhamnaceae, Urticaceae and Solanaceae.[45-47]

Glioma cell growth inhibition

The results indicated potential activity in murine glioma cell lines inhibitory for a DP sample of *T. angustula*, and they are the consequence of the presence of phytoactive compounds in its chemical composition. The *in vitro* cytotoxic activity on the glioma cell line studied, dose-dependent, determined the minimum cytotoxic concentration (MCC), based on the statistically significant difference ($p < 0.05$) between relative survival (%) and the control group (DMEM + dimethyl sulfoxide (DMSO), the sample solvent) by the MTT test. The results showed that the median MCC was 1000 ng mL^{-1} honey (range: 100-15000 ng mL^{-1}), with a 16.19% difference in the control group. In a review study, Waheed *et al*.,[52] gathered information about the anticancer mechanisms of action of honey and highlight the main constituents probably responsible for this potential.

Exploratory analysis

To better understand the data set, the interrelationships and differences between DP and RP samples, the multivariate principal component analysis (PCA) was applied to evaluate the physicochemical parameters (pH,

Table 1. Phenolic compounds reported in *Tetragonisca angustula* honey in different Brazilian regions from floral origin

Sample	Phenolic compounds						Floral origin	Reference
	Caffeic acid	Chlorogenic acid	*p*-Coumaric acid	*trans*-Ferulic acid	Rutin	Quercetin		
A / (μg 100 g^{-1} of honey)	20.0 ± 0.01	n.d.	28.4 ± 0.03	7.00 ± 0.02	n.a.	5.00 ± 0.01	no information	Bilula *et al.*[43]
B / (mg 100 g^{-1} of honey)	n.d.	n.d.	0.044 ± 0.01	0.018 ± 0.01	n.a.	0.032 ± 0.01	sylvan	Biluca *et al.*[49]
C / (mg 100 g^{-1} of honey)	n.d.	n.d.	0.006 ± 0.1	n.d.		0.006 ± 0.01		Biluca *et al.*[49]
D / (μg 100 mg^{-1} of honey)	1.77 ± 0.1	< LOQ	inconclusive	inconclusive	0.94 ± 0.01	n.d.	*Spondias tuberosa*; *Mimosa tenuiflora*; *Ziziphus joazeiro*; *Schinus terebinthifolius*	this study

Mean values of three analyses of three different samples ± standard deviations; LOQ: limit of quantification; n.d.: not detected; n.a.: not analyzed.

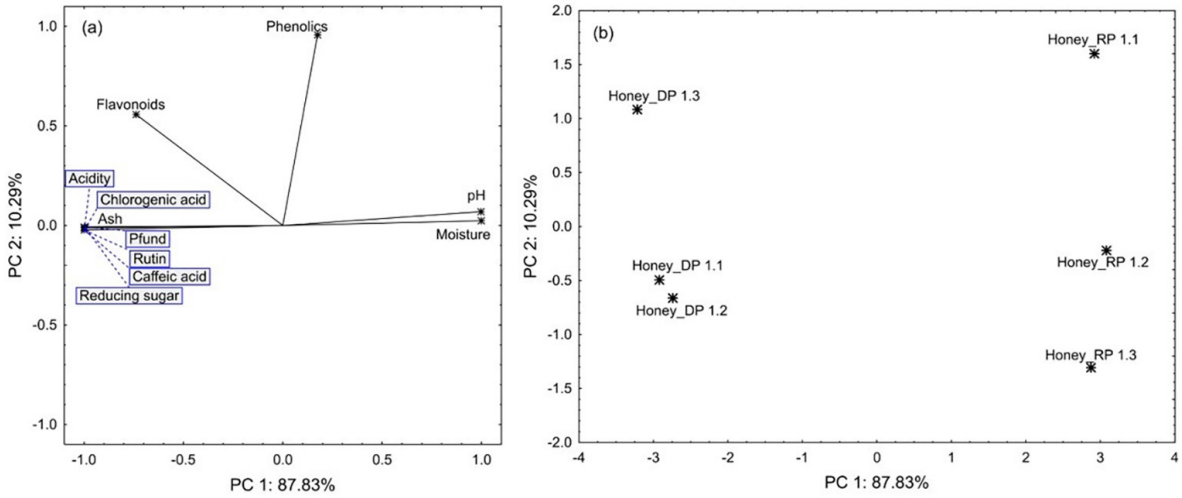

Figure 3. First principal component *versus* second principal component (PC1 × PC2). (a) Distribution of variables on loadings plot and (b) distribution of samples (DP: dry period; RP: rainy period) on scores plot.

acidity, moisture, reducing sugars, ash content and color), flavonoid content and phenolic compounds. PCA is a very useful technique for visualizing the natural sample distribution in a reduced dimensional space.[53] PCA is an unsupervised technique that reduces the dimensionality of the experimental data by grouping the correlated information into the same PC.[54]

The graphs obtained by PCA are presented in Figure 3. The distribution of variables (loadings plot) (Figure 3a) and the distribution of samples (scores plot) (Figure 3b), using the combination PC1 *versus* PC2 were obtained. Table S1 (Supplementary Information section) shows the results obtained by the DP and RP samples.

According to the PCA analysis, the first two PCs (PC1 and PC2) accounted for 98.12% of the data variance. All variables analyzed, with the exception of phenolic compounds, are explained in PC1 and describe 87.83% of the total variance. The second PC is described only by the variable phenolic compounds, with 10.29% of the total variance. The high percentage achieved with PCs confirms the effectiveness of PCA to distinguish honey samples according to physicochemical parameters. Loadings values for the first three PCs are shown in Table S1 in the SI section.

The PC1 × PC2 graph allowed to confirm the statistically significant and evident difference between samples DP and RP, as there was a grouping of samples from the same periods and clear separation between samples from different periods of collection (Figure 3b). The loading plot (Figure 3a) accurately demonstrated the variables responsible for differentiating the samples.

The physicochemical characteristics were the main variables responsible for the difference between DP and RP samples, in which pH and moisture stand out in the justifiable similarity between RP samples and it was crucial to show their difference with DP samples. The other characteristics: acidity, reducing sugars, ash content and color were relevant to propose the similarity between DP samples and distinguish them from RP samples.

Conclusions

Our study highlights the difference in the physicochemical profile of honeybee *T. angustula* (Jataí) collected in dry and rainy periods in the semiarid region of Bahia, Brazil and, nevertheless, similarities in the chemical composition of these samples were observed. The analysis by PCA allowed to verify distinct profiles between honey samples and indicates the physicochemical parameters as the variables mostly responsible for this difference. Such variations are justified by the climatic characteristics and floristic variability in the different periods of the year in the Southwestern region of Bahia.

Despite the difference in edaphoclimatic factors and floristic availability in the two analyzed periods, the results do not show consistent evidence to admit an influence on the content of phenolic compounds and flavonoids between the DP and RP. Considering the conditions studied, DP honey samples present *in vitro* evaluation of the inhibition property on glioma cell growth at low concentration, and this property is generally associated with the bioactive compounds of honey. To our knowledge, this research is pioneering in quantifying phenolic compounds in *T. angustula* honey in different climatic periods in this region.

Supplementary Information

Supplementary data (Table S1) are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), Finance Code 001, and Universidade Estadual do Sudoeste da Bahia (UESB).

Author Contributions

ABF, JMPB and AJP monitored the collection of honey samples and carried out the physicochemical characterization procedures. ABF, DMO and HOJ followed the spectrophotometric tests for the determination of flavonoids and phenolic compounds and quantification by HPLC. JMPB and FSR followed the performance of the murine glioma cell inhibitory effect. ABF, RMA and CGN followed all the stages of the study, as well as performed the statistical treatment, including PCA. All authors have approved the final manuscript.

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Submitted: March 1, 2022
Published online: July 5, 2022